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The role of tocopherol cyclase in salt stress tolerance of rice (*Oryza sativa*)

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Tocopherols synthesized exclusively by photosynthetic organisms are major antioxidants in biomembranes. In plants, tocopherol cyclase (TC/VTE1) catalyzes the conversion of 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) to γ-tocopherol. In the present study, *OsVTE1*, which encodes a rice tocopherol cyclase ortholog, was cloned and characterized. *OsVTE1* was induced significantly by abiotic stresses such as high salt, H_2O_2, drought, cold and by the plant hormones ABA and salicylic acid. The tissue-specific expression pattern and *OsVTE1*-promoter GUS activity assay showed that *OsVTE1* was mainly expressed in the leaf, and also could be detected in the root, stem and panicle. Compared with control plants, transgenic plants with *OsVTE1* RNA interference (*OsVTE1*-RNAi) were more sensitive to salt stress whereas, in contrast, transgenic plants overexpressing *OsVTE1* (*OsVTE1*-OX) showed higher tolerance to salt stress. The DAB *in vivo* staining showed that *OsVTE1*-OX plants accumulated less H_2O_2 than did control plants.

rice, tocopherol, tocopherol cyclase (TC/VTE1), transgenic rice, abiotic stress tolerance

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Tocopherols and tocotrienols, collectively known as vitamin E, are amphiphilic lipids and are synthesized exclusively by the photosynthetic organisms [1]. Vitamin E is an essential component of the human diet and performs numerous critical functions including quenching and scavenging various reactive oxygen species (ROS) and free radicals and protecting polyunsaturated fatty acids from lipid peroxidation [2,3]. Tocopherols are composed of a polar chromanol head and a lipophilic isoprenoid tail derived from homogentisate and phytyl diphosphate, respectively [4]. In membrane lipid bilayers, the polar chromanol head is exposed to the surface of the membrane and the lipophilic isoprenoid tail associates with lipids. In nature there are four forms of tocopherols, namely α-, β-, γ- and δ-tocopherol, differing only in the number and position of methyl substituents attached to the chromanol ring. Plant tissues vary enormously in their contents and compositions of tocopherols. While α-tocopherol is the predominant form in leaves, γ-tocopherol is rich in seeds of many plant species [5,6].

Because of its high economic value, substantial efforts have been devoted to elucidating the tocopherol biosynthetic pathway in plants and cyanobacteria in recent years [7,8]. Tocopherol cyclase (VTE1) catalyzes the penultimate step of tocopherol synthesis, converting the substrate 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) to γ-tocopherol [8–10]. Final methylation by γ-tocopherol methyltransferase (γ-TMT) results in the production of α-tocopherol [11]. TC/VTE1 activity is evolutionarily conserved

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between plants and cyanobacteria [8], and is a major limiting factor of tocopherol synthesis in leaves of *Arabidopsis thaliana* as well [12].

Under natural conditions, plants are constantly challenged by a variety of biotic and abiotic stresses. High salinity is one of the major abiotic stresses that severely reduce plant growth and crop production. An important response to the high salinity stress is the accelerated generation and/or accumulation of ROS including hydrogen peroxide, hydroxyl radicals, and superoxide anions, which could damage the cellular components or even cause cell death [13–17]. To deal with oxidative stress, plants utilize enzymatic and non-enzymatic antioxidants, of which the latter includes vitamin E, to protect their cells from oxidative damage by scavenging ROS [18,19].

Previous studies have shown that oxidative stress activated the expression of genes responsible for the synthesis of tocopherols in higher plants and water deficiency resulted in an increase of tocopherol concentration in plant tissues [20–22]. In addition, the concentration of α-tocopherol precursor and abiotic stress tolerance are correlated [23]. In transgenic lettuce plants overexpressing *Arabidopsis TC/VTE1*, the chlorophyll content increased by up to 35% [24] and transgenic tobacco plants overexpressing *Arabidopsis TC/VTE1* showed enhanced tolerance to drought stress [25].

However, to our knowledge, roles for tocopherols in the response to high salt stress in rice have not been clearly demonstrated. In the present study, we conducted research on the responses of *OsVTE1* knockdown and overexpression of transgenic lines to high salinity to assess functions of tocopherols in rice. The results showed that overexpression of *OsVTE1* could increase the tolerance to salt stress by efficiently scavenging ROS in rice.

1 Materials and methods

1.1 Growth conditions

Seeds of rice (*Oryza sativa* subsp. *japonica* cv. TP309), and transgenic plants were sown in pots (8 cm×10 cm) containing vermiculite soaked with water. All plants were grown under white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h photoperiod) and 75% relative humidity at 28°C in a greenhouse.

1.2 RNA isolation and RT-PCR and real-time quantitative RT-PCR analysis

Total RNA isolation was performed following the method described by Zhang *et al*. [26]. First-strand cDNA synthesis was primed with Oligo$(dT)_{15}$ and catalyzed with M-MLV reverse transcriptase (Promega) at 37°C for 1.5 h. A five-fold dilution of the reaction products was used as templates for RT-PCR and real-time quantitative RT-PCR analysis. The gene-specific primers RT-OsVTE1 (5′-AGGGCCTATTCATCTCTACC-3′) and RT-OsVTE2 (5′-GGTGTCCATTCCCGAGTGCAGGCA-3′) were used for RT-PCR. Real-time quantitative RT-PCR analysis used the SYBR Green PCR Master Mix, an ABI 7000 sequence detection system (Applied Biosystems), and the gene-specific primers Rtime1 (5′-TGCAATGTCTTCTCAGGCGC-3′) and Rtime2 (5′-GCTTCTATTTCAACCAGATG-3′). Quantitative RT-PCR results were analyzed with Microsoft Excel software.

1.3 Vector construction and rice transformation

The full-length cDNA sequence of *OsVTE1* (Os02g0276500) was amplified by RT-PCR using gene-specific primer pairs (5′-CGGGGTACCAGGGCCTATTCATCTCTACC-3′ and 5′-CGCGGATCCAGCATCAGCATGGACCTCGC-3′), and cloned into the *Kpn* I and *Bam*H I sites of the binary vector pBIN438 as described previously [27]. The gene was driven by two copies of the 35S promoter. The tobacco mosaic virus Ω sequence was also included downstream of the 35S promoter to enhance the translation efficiency. The construct was introduced into *Agrobacterium tumefaciens* strain AGL1 and then transformed into rice (*O. sativa* subsp. *japonica* cv. TP309) as described by Hiei *et al*. [28]. For the construction of the RNAi vector pZH*OsVTE1*, a 489-bp fragment of the *OsVTE1* gene (from 409 to 897 bp) was amplified using the specific primers RNAiF containing the *Xba* I and *Sac* I sites (5′-TGCTCTAGAGAGCTCCAGTTCACCGAGAAATCC-3′) and RNAiR containing the *Sal* I and *Sac* I sites (5′-ACCGTCGACGAGCTCAGATGCGCCTGAGAAGAC-3′). The sense and antisense fragments were assembled into vector pZH01. The RNAi construct pZH*OsVTE1* contains the 35S promoter, the *NOS* terminator, and a fragment of the *GUS* gene between the two inserted 489-bp fragments of the *OsVTE1* gene.

1.4 H_2O_2 localization *in situ*

Plant leaves were excised and immersed in a 1% solution of 3′,3′-diamino benzidine (DAB) in Tris-HCl buffer (pH 6.5). After vacuum-infiltration for 30 min, the samples were incubated at room temperature for 20 h in the dark. When the brown spots were seen clearly, leaves were bleached by immersion in boiling ethanol to visualize the brown spots. The brown spots were characteristic of the reaction of DAB with H_2O_2.

1.5 Determination of the total chlorophyll content

About 0.1 g leaf tissue excised into segments 2–3 cm in length was immersed in the extract solution (45% ethanol:45% acetone:10% water, V/V) at room temperature until the leaves were bleached. The absorbance of the extracts was measured at 647 nm and 665 nm. The total chlorophyll

content was calculated [29] and expressed as mg g^{-1} FW. Three replicate experiments were performed.

1.6 Promoter GUS analysis

The *OsVTE1* gene promoter (DNA fragment from 1 to 1300 bp upstream of the translation start site) was amplified using the primers 5′-CCAAGCTTGCACGACCATAGGCGTGG-GT-3′ and 5′-GCTCTAGAGCTGATGCTGCGGGCGGG-CA-3′, confirmed by sequencing and cloned into the *Hin*d III and *Bam*H I sites of vector pBI121. The plasmid was introduced into the receptor rice (*O. sativa* subsp. *japonica* cv. TP309) by *A. tumefaciens*-mediated transformation as described by Hiei *et al*. [28]. The GUS assays at different developmental stages were performed according to the method described by Jefferson *et al*. [30].

1.7 Salt treatments

All three-week-old seedlings including the control and transgenic plants grown under normal conditions were transferred into 100 mmol L^{-1} NaCl solution for 10 d, rinsed three times with water and then subjected to regular irrigation for recovery. Three replicate experiments were performed.

2 Results

2.1 Expression of *OsVTE1*

The full-length *OsVTE1* (Os02g0276500) cDNA was amplified using gene-specific primers by RT-PCR. *OsVTE1* had an open reading frame (ORF) of 1413 bp and encoded a peptide of 471 amino acids. Sequence alignment analysis indicated that OsVTE1 exhibited 83.9% similarity with SXD1 in *Zea mays* (GenBank accession number AAK60502) and 61.9% with VTE1 in *Arabidopsis thaliana* (accession number NP_567906) (Figure 1).

The expression pattern of *OsVTE1* under various treatments was investigated by RT-PCR. It could be seen in Figure 2A that *OsVTE1* was induced significantly by abiotic stresses including 200 mmol L^{-1} NaCl, drought, –4°C, 100 μmol L^{-1} H_2O_2, 100 μmol L^{-1} salicylic acid (SA), and 100 μmol L^{-1} abscisic acid (ABA). However, *OsVTE1* expression in response to 100 μmol L^{-1} ACC and H_2O treatment was unchanged. The results suggest that *OsVTE1* may be involved in the tolerance of rice to changes in the environment.

Total RNA was extracted from the root, stem, leaf, and spikelet of rice TP309 to study the tissue-specific expression pattern of *OsVTE1*. As shown in Figure 2B, *OsVTE1* was mainly expressed in the leaf, and also could be detected in the root, stem and panicle. To further examine the expression of *OsVTE1*, the 1.3 kb promoter region of *OsVTE1* was cloned and fused to the expression vector pBI121 containing β-glucuronidase (*GUS*) reporter gene (Figure 3C). The resulting construct was transferred into rice TP309. Fifteen independent T_2 transgenic lines were used for the GUS staining assay. Consistent with the results obtained from the RT-PCR assay mentioned above, GUS driven by the *OsVTE1* promoter was expressed in the stem, especially in the node, spikelet, particularly in the stamen, and leaf as indicated in Figure 2C (a–h).

2.2 Phenotype analysis of transgenic plants under salt stress

An overexpression vector with the full-length cDNA of

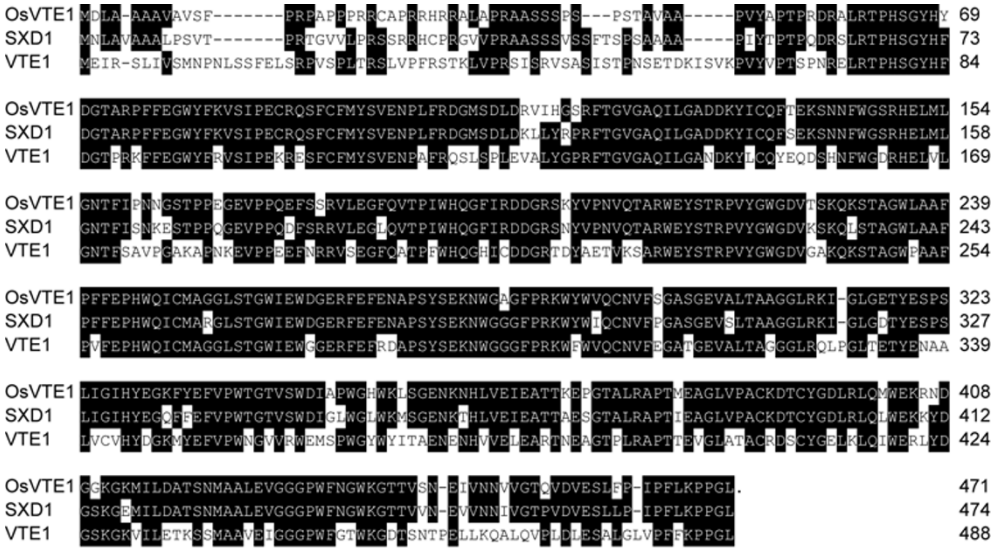

Figure 1 Alignment of the deduced amino acid sequences of OsVTE1, SXD1 in *Zea mays* (accession number AAK60502) and VTE1 in *Arabidopsis thaliana*. Alignments were performed using DNA STAR. Consensus amino acid residues are shaded in black. Gaps introduced to maximize the alignments are denoted by hyphens.

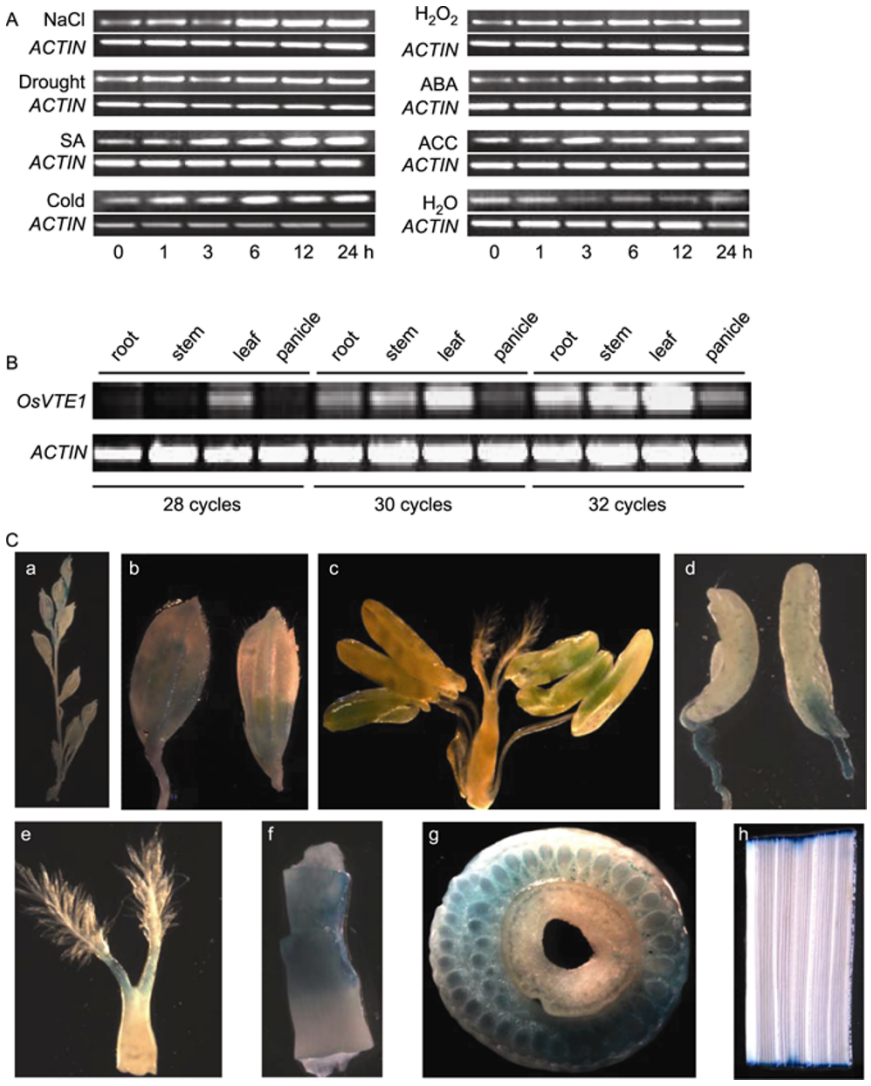

Figure 2 Expression of *OsVTE1* in rice. A, Expression of *OsVTE1* in rice seedlings in response to salt, H_2O_2, drought, ABA, SA, ACC and cold treatments. B, Expression of *OsVTE1* in the root, stem, leaf, and spikelet determined by 28, 30, and 32 cycles of RT-PCR with gene-specific primers. C, *OsVTE1* promoter-*GUS* expression in rice plants: spikelet (a), seed (b), rachilla (c), stamen (d), pistil (e), stem (f, g), and leaf (h).

OsVTE1 under the control of two CaMV35S promoters (*OsVTE1*-OX) and a RNA interference vector carrying part of the *OsVTE1* gene (*OsVTE1*-RNAi) (Figure 3A and B) were constructed and transformed into the TP309 plants. The expression of *OsVTE1* was checked for the *OsVTE1*-OX and *OsVTE1*-RNAi transgenic plants by real-time PCR (Figure 3D and E). *OsVTE1* gene expression in six of the 11 *OsVTE1*-OX transgenic lines was higher than that of wild-type TP309. By contrast, *OsVTE1* gene expression in most of the *OsVTE1*-RNAi transgenic plants was lower than that of wild-type TP309. The progeny of six independent lines *OsVTE1*-OX-14-2, 20-3, 75 and *OsVTE1*-RNAi-3-2, 13-3, 64 were used for further analysis. No visible phenotypic differences were observed between transgenic plants and control plants when grown under normal conditions (Figure 4A).

Given that the expression of *OsVTE1* was induced by NaCl treatment, the phenotypes of the *OsVTE1* transgenic plants were compared with those of control plants under the salt treatment. After growth under normal conditions for 3 weeks, all of the transgenic and control plants were transferred to 100 mmol L^{-1} NaCl solution for salt treatment and the phenotypes were observed after 10 d. Nearly 80% of the leaves of the 3 *OsVTE1*-RNAi lines were wilted. The control plants were short, yet the leaves were still green. The growth of *OsVTE1*-OX plants was much better than that of the controls (Figure 4B).

After treatment for 10 d, all plants were rinsed 3 times with water, then subjected to regular irrigation for recovery. As shown in Figure 4C and E, the *OsVTE1*-OX plants recovered more quickly than the others and about 60%–80% of them could survive after resuming irrigation for 10 d. In contrast, the survival rate of the control plants *TP309* was about 40%. The lowest survival rate was seen in the *OsVTE1*-RNAi

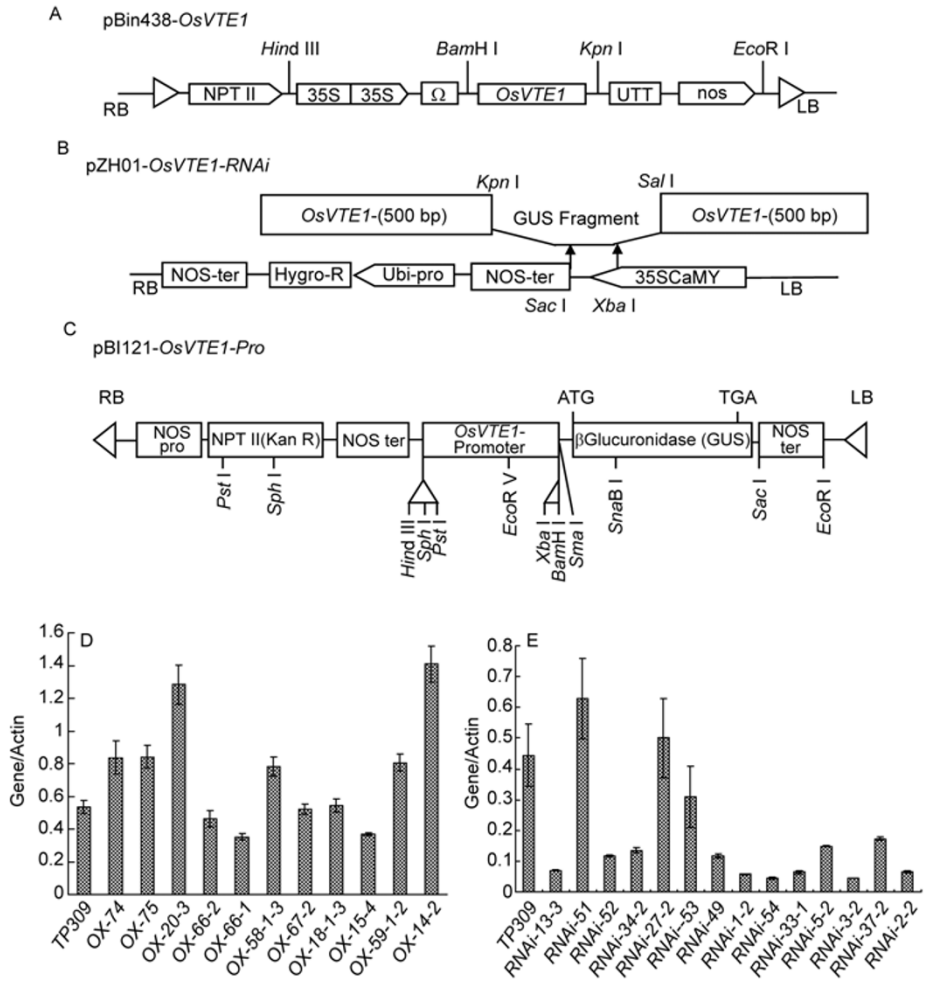

Figure 3 Transgenic plant materials. A, Schematic diagram of overexpression vector pBin438-*OsVTE1*. B, Schematic diagram of RNA interference vector pZH01-*OsVTE1-RNAi*. C, Schematic diagram of promoter GUS vector pBI121-*OsVTE1*-promoter. D, Expression of *OsVTE1* in overexpression plants determined by real-time PCR. E, Expression of *OsVTE1* in RNA interference plants determined by real-time PCR.

plants; most of the plants died and only about 30% of them survived.

Leaf chlorophyll content provides valuable information about the physiological status of plants and may be used as an indicator of photosynthetic activity [31]. Changes in chlorophyll content can occur as a result of exposure to environmental stresses during growth. Figure 4D shows that the chlorophyll content in all plants decreased after salt stress, though in different degrees. For the *OsVTE1*-RNAi plants, the chlorophyll content was reduced by about 50%–60%, compared with about 40% in the control plants and about 26% in the *OsVTE1*-OX plants.

2.3 Overexpression of *OsVTE1* increased the antioxidant capacity of rice seedlings

Salt stress can induce the accumulation of ROS such as H_2O_2 [32]. After treatment with 100 mmol L^{-1} NaCl for 10 d, the leaves of three-week-old rice seedlings were excised. Accumulation of H_2O_2 was evaluated *in situ* by histochemical detection with 3,3′-diaminobenzidine (DAB) staining. As shown in Figure 5, salt stress produced very little H_2O_2 brown spots in the *OsVTE1*-OX plants. Half of the leaves of the TP309 plants became brown under salt stress. More serious damage was observed in the *OsVTE1*-RNAi plants, in which almost the whole leaf became brown. These results indicated that overexpression of *OsVTE1* might be able to efficiently eliminate H_2O_2 produced by salt stress.

3 Discussion

Among the characterized functions of tocopherols in cells, scavenging and quenching ROS and lipid-soluble byproducts of oxidative stress was the most predominant [2,33,34]. Salt stress could induce the accumulation of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals [32]. At the same time, the amounts of various antioxidants including tocopherols increased strongly to scavenge ROS. Based on the studies of 10 different grass species, it was

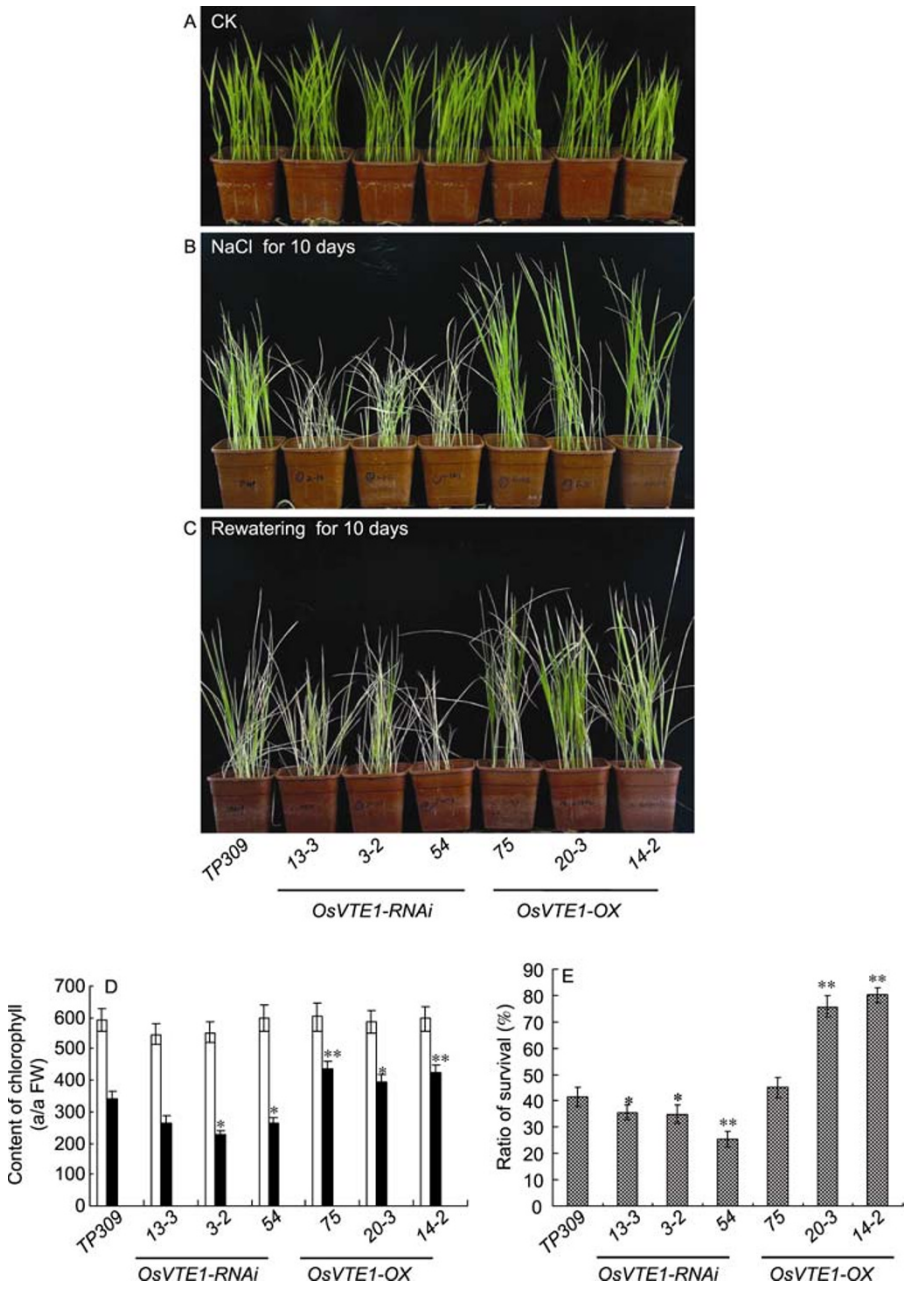

Figure 4 Salt stress tolerance of the OsVTE1-RNAi and OsVTE1-OX transgenic plants. The stress treatments were conducted using plants growing under normal conditions for 3 weeks. A, Normal conditions for 3 weeks. B, 100 mmol L^{-1} NaCl stress for 10 d. C, 10-day-recovery after 100 mmol L^{-1} NaCl stress for 10 d. D, The chlorophyll content after 100 mmol L^{-1} NaCl treatment for 10 d. White represents CK, black represents NaCl treatment. Each column represents an average of three replicates, and bars indicate the SD. E, The ratio of survival after 10 d of recovery. Each column represents an average of three replicates, and bars indicate the SD. * and ** indicate significant differences from the corresponding control plants in the same treatments at $P < 0.05$ and $P < 0.01$, respectively.

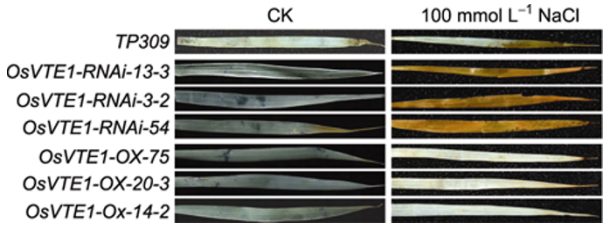

Figure 5 DAB (3,3-diaminobenzidine) staining after 100 mmol L^{-1} NaCl stress for 10 d.

reported that drought stress led to an increase in tocopherol concentration of one- to three-fold in nine of the 10 species and highly significant correlations were observed between stress tolerance and tocopherol concentration [21,23]. The accumulation of antioxidants under stress was at least partially due to induction of gene expression. In *Arabidopsis*, *HPPD* (p-hydroxyphenylpyruvate dioxygenase) and *HPT1* (homogentisate phytyltransferase), two genes encoding enzymes involved in tocopherol synthesis, were induced under

light stress [35]. In contrast, the expression level of *AtVTE1* was not altered significantly during stress in *Arabidopsis* [35]. In *Arabidopsis*, experiments using leaf discs from two vitamin E mutants, a tocopherol cyclase mutant (*vte1*) and a homogentisate phytyl transferase mutant (*vte2*), indicated that tocopherols function as protectors of membrane lipids against peroxidative damage under high light intensity combined with low temperature conditions [36]. Our results demonstrated that different expression levels of *OsVTE1* had a strong impact on the loss of chlorophyll content under high salt stress in rice. In this regard, we could conclude that *OsVTE1* plays an important role in protecting the photosynthesis system in rice when exposed to salt stress.

Manipulation of tocopherol content has been reported in *Arabidopsis* by overexpressing the genes encoding the enzymes for tocopherol biosynthesis. Overexpression of *HPT/VTE2* and *TC/VTE1* increased levels of tocopherols up to 4.4- and 7-fold, respectively, in *Arabidopsis* leaves [12,37]. To study the impact of tocopherol content on abiotic stress tolerance in rice, we produced both *OsVTE1* overexpressed and knocked-down rice transgenic plants. The results indicated that overexpression of *OsVTE1* could eliminate H_2O_2 efficiently in rice. On the contrary, knock-down of *OsVTE1* resulted in a significant increase of H_2O_2 amounts under salt stress (Figure 5). The salt stress tolerance testing showed that the plants overexpressing *OsVTE1* had higher salt tolerance than control and *OsVTE1*-RNAi plants (Figure 4). It is suggested that enhancement of the tocopherol level might be related to the function of preventing lipid peroxidation and scavenging ROS.

In plants, tocopherol composition varies between different tissues within a species. Generally, α-tocopherol accumulates mainly in leaves, and seeds are rich in γ-tocopherol. β- and δ-tocopherol are not very abundant in most plants species. α-tocopherol together with other hydrophilic antioxidants glutathione and ascorbate has been proposed to participate in the scavenging of ROS [38]. By comparing the effects of a general tocopherol deficiency (in HPT:RNAi) versus a replacement of α-tocopherol with γ-tocopherol (in γ-TMT:RNAi) in oxidative stress scenarios, Abbasi *et al*. [39] reported that α- and γ-tocopherol played specific roles in abiotic stress responses of transgenic tobacco. However, from the report by Kruk *et al*. [40], the inhibitory effect of interrupted tocopherol biosynthesis on singlet oxygen scavenging in PSII of *Chlamydomonas reinhardtii* could be overcome by adding exogenous membrane-permeable short chain α- and γ-tocopherol derivatives, indicating that α-tocopherol could be substituted by γ-tocopherol in leaves. Biochemical analysis of an *Arabidopsis* plant (vte4-1) carrying a functional null mutation in the γ-TMT gene showed similar results. The mutant accumulated high levels of γ-tocopherol instead of α-tocopherol. α-tocopherol can be replaced by γ-tocopherol in vte4-1 to protect the photosynthetic apparatus against oxidative stress [41].

In summary, we have reported that *OsVTE1*, which encodes atocopherol cyclase, was induced by various abiotic stresses. *OsVTE1*-OX plants accumulated less H_2O_2 than did control plants. This result, together with the phenotype analysis under salt stress, suggested that over-expressing *OsVTE1* could improve the tolerance to high salt stress in rice. A future goal is to measure tocopherol content of transgenic rice lines overexpressing and RNA-interfering *OsVTE1* and further verify the relationships among *OsVTE1* expression, tocopherol content and rice performance under salt stress.

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